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dHPLC Method for Forensic DNA Analysis

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Executive Summary

Introduction

Forensic DNA testing is seriously hampered by a growing backlog of DNA samples. There are more than a half million unsolved criminal cases awaiting DNA testing. Part of the solution is the development of new analytical methods to reduce the backlog. High accuracy of the results of DNA testing is of critical importance. Analysis time and expense are also significantly important considerations. New methods under development for reducing the backlog are based on applications of denaturing high-performance liquid chromatography (dHPLC).

Purpose of the report

The overall goal of the work was to contribute to forensic DNA research and development by generating results and findings that are useful to crime laboratories. The scope of the work was to assess the potential for dHPLC for development into a useful analytical method for forensic DNA testing. The project evaluated whether dHPLC has the potential of being developed into a useful analytical method for DNA testing. Exploratory studies were performed on the extension of dHPLC techniques to the detection and characterization of modified DNA.

Forensic applications of Denaturing High-Performance Liquid Chromatography (dHPLC)

dHPLC is a recently developed specialized version of high-performance liquid chromatography (HPLC). Whereas HPLC is a standard piece of equipment used in many crime labs for trace and drug analysis, dHPLC is currently available in only a few labs. The advantage of dHPLC over HPLC is applications in DNA testing. Current dHPLC methods can detect DNA Single Nucleotide Polymorphisms (SNPs) in less than ten minutes following PCR amplification. The methodology for obtaining sample DNA is standard. The researchers collected hair samples from fifty volunteers. By sampling the root, they were able to acquire DNA. Experiments were conducted using dHPLC to detect SNPs from mitochondrial DNA. Although further work is needed to develop a standardized method for forensic DNA analysis, the researchers concluded that dHPLC is a promising way to analyze DNA with potentially significant advantages over commonly used methods.

Current DNA analytical methods are most effective for detecting and identifying unmodified DNA. New methods for detecting modified DNA would be useful to crime labs, because detection of specifically-modified DNA could assist in the identification of individuals exposed to certain environmental, occupational and dietary exposures to a variety of genotoxic chemicals. Exposure biomarkers for genotoxic substances are being rapidly developed for disease states and for safety considerations. Forensic applications will become obvious once the biomarkers are developed and validated. As an extension of our work on dHPLC methods development for DNA, we applied dHPLC as a separation tool for isolation of modified DNA by mass spectrometry in a preliminary study. In these experiments, a small piece of duplex DNA was reacted with a metabolite related to methamphetamine and the products separated by dHPLC. The results of mass spectrometric analysis of the products showed that dHPLC has good potential as a separation method for modified DNA. Future work is planned to develop a forensic method for determination of smoked methamphetamine based on modified DNA biomarkers.

Conclusions and Implications

SNP analysis by dHPLC is a useful screen of DNA samples prior to sequencing. If the current dHPLC methods are widely applied, significant savings of time and money are realistically possible. The backlog of the most important DNA samples would be significantly reduced if time-saving methods that use dHPLC were used by crime labs.

Strengths of dHPLC methods include ease of instrument operation, high sample throughput, shorter sample analysis time, and potential for new methods development. Commercially available dHPLC instrumentation is as easy to use as are standard HPLC instruments. High throughput dHPLC is readily available. The cost per sample is low in comparison with sequencing. dHPLC sample analysis time is less than ten minutes, which is much shorter time than the PCR step in the analysis. dHPLC is a flexible platform for development of new applications in DNA testing.

Weaknesses include the absence of robust methods that can be routinely applied by crime labs and training needs. There is a potential issue regarding the robustness of the analytical dHPLC methods when applied broadly in a variety of settings. The level of expertise and training needed to maintain and operate currently available dHPLC instrumentation is the same as that needed for HPLC. Standardized procedures are not available that can be used in crime labs with existing equipment and varying levels of personnel qualifications. Intra- and interlaboratory studies using a standard method have not been conducted to validate the method.

New methods for modified DNA is an area of future application of dHPLC technology, especially when used in conjunction with mass spectrometric techniques. Detection of a chemical in a criminal exposed to that same chemical at a crime scene can make a good case for placing the criminal at the scene. Timing in the detection of the material is often critical since most chemicals are rapidly eliminated from the body. Some chemicals

persist when bound to DNA or proteins. Examples of chemicals that bind strongly include electrophiles, such as some tear gas components. Some electrophiles are generated in the body by the action of drug metabolizing enzymes. From another project in our laboratories, we found that a breakdown product formed from smoking methamphetamine is converted by liver enzymes to an electrophilic epoxide that reacts with DNA. We found that dHPLC provided an effective method for separating adducts formed from the parent oligonucleotide. Out results support an increased role for dHPLC and mass spectrometry in the development of forensic biomarkers.

Project Description

Background

dHPLC is fast becoming an important technique for DNA analysis. For excellent review articles on dHPLC, see references 1 and 2, and for a comprehensive bibliography on dHPLC, see the Stanford Genome Technology Center DNA Variation Group website at http://insertion.stanford.edu/pub.html. The clinical research community reports that dHPLC provides new methods for detecting DNA biomarkers for cancer and other diseases (3-5), and mutation screening (6-13). Betz and coworkers found that dHPLC is a useful prescreening method for detecting aberrant DNA methylation in cancer (11). dHPLC methods are being developed for SNP analysis (2, 14-16). These applications to SNP detection are a resource for the development and validation of forensically relevant methods. dHPLC has played a role in detecting biomarkers of genotoxic effects and cancer (17-20)

Published forensic applications of dHPLC include a comparison of dHPLC with direct sequencing for SNP detection by Shi, *et al.* (21), studies by Shinak *et al.* (22) on a method for sex identification by heteroduplex analysis, and the work of LaBerge, Shelton and Danielson on the development of a dHPLC method for mitochondrial DNA analysis (23).

The dHPLC platform is ideal for the separation of Polymerase Chain Reaction (PCR) amplified SNPs. PCR is a process by which minute amounts of DNA (as from a single strand of hair) are amplified several hundred times to yield specific stretches of DNA that can be analyzed using techniques such as gel electrophoresis, direct sequencing or the more recently proposed technique of dHPLC. In dHPLC, the amplified PCR products are separated on a patented chromatography column to produce distinct DNA information for each individual. We proposed to evaluate this novel, easy and rapid technique of DNA analysis as a candidate method for forensic DNA testing.

Applications of dHPLC methods are under consideration as a means to identifiy individuals through DNA analysis accurately and more quickly than can be done by current methods.

Scope and Methodology

DNA Extractions: DNA was extracted from hair shaft material using Promega® Tissue and Hair Extraction kit in concert with DNA IQ™.

IRB Approval: Approval from the West Virginia University IRB was obtained for collection of human tissue to use as samples of DNA. DNA was extracted from the hair of 35 individuals using the Tissue and Hair Extraction kit in concert with DNA IQ™ (Promega; Madison WI). Attempts to isolate DNA from nail clippings were unsuccessful.

PCR: 5 µl of total extracted DNA was added to PCR reactions containing Amplitaq Gold® Buffer, Amplitaq Gold® DNA Polymerase (1.25 U/reaction), 2.5 mM $MgCl_2$ 200 µM each dNTP, 0.4 µM each primer, 50 µl total reaction volume. Multiplex samples were the same except for adjustment of the primer.

Primers: The primers used for mt DNA hypervariable studies are listed below (24).

HVI Fwd 5'- CAC CAT TAG CAC CCA AAG CT -3' 15977
Rev 5'- GAG GAT GGT GGT CAA GGG AC -3' 16410

HVII Fwd 5'- CTC ACG GGA GCT CTC CAT GC -3' 28
Rev 5'- CTG TTA AAA GTG CAT ACC GCC A -3' 429

The SNP Primers from Vallone *et al.* (25) were:

4580 Fwd 5'- TCT TTG CAG GCA CAC TCA TG -3'
5004 Rev 5'- TGG TTA TGT TAG GGT TGT ACC G -3'

7028 Fwd 5'- GGC CTG ACT GGC ATT GTA TT -3'
7202 Rev 5'- TTG ATG TGG TGT ATG CAT CG -3'

dHPLC Analysis: Non-denaturing conditions (Varian universal mth. 50) were used to evaluate PCR product specificity at 50°C. A puc/Hae III digest was used as the standard, positive control. Injection volumes were 5 µl and controls were run as initial and final samples. In addition, reagent blanks were used to determine the presence of any possible column contamination. PCR reactions have been optimized in order to produce consistent peak area from both uniplex and multiplex reactions.

Heteroduplex formation: PCR products were mixed in a 1:1 ratio based on peak area obtained from non-denaturing dHPLC analysis of PCR products. The mixtures were then heated at 94°C for 4 min, then slowly cooled to 25°C @ ~ 1.5°C/min in order to allow for heteroduplex formation to occur.

dHPLC analysis of hetero- and homo duplex formation: Optimal temperatures for each of the four uniplex amplicons were first estimated empirically using the program available @ http://insertion.stanford.edu. In order establish optimal temperatures, all PCR amplicons were screened at 56°C, 57°C, 58°C, 59°C and 60°C using corresponding Varian Universal method. Controls were Zero DNA template PCR reaction, reagent blank (tests for column contamination). DYS271 mutation standard as a positive control was run first and last to monitor column performance.

dHPLC Instrumentation: The dHPLC system was a Varian Helix system consisting of a proprietary Helix DNA column and solvents, ProStar 430 autosampler, dual ProStar 210 solvent delivery modules, ProStar 520 column oven, ProStar 340 UV-Vis detector, and Star Reviewer software.

Mass Spectrometry: Mass spectra were obtained on a Thermo Finnigan LCQ DECA ion trap mass spectrometer operating under electrospray ionization in the negative ion detection mode.

Results

Non-denaturing conditions (Varian universal mth. 50) were used to evaluate PCR product specificity at 50°C. A puc/Hae III digest was used as standard/positive control. Injection volumes were 5 µl and controls were run as initial and final samples. In addition, reagent blanks were used to determine the presence of any possible column contamination. PCR reactions have been optimized in order to produce consistent peak areas from both uniplex and multiplex reactions.

Mitochondrial DNA was chosen as the initial DNA source for study. Limitations regarding the use of mtDNA for definitive identification do exist. The small size of the mtDNA genome (16,569 bp) does not allow for the power of discrimination obtained through genomic STR analysis. In addition since mtDNA is primarily inherited via maternal linkage, it is not possible to discern between maternal siblings. Earlier work has provided strong evidence of the effectiveness of dHPLC in a forensic setting (23). The work focused on Hypervariable regions I and II which may not provide sufficient discriminatory power to identify individuals. This is apparent when one considers that 7% of the caucasians display the same HV haplotype. Certain SNPs can be identified to increase the discriminatory power of mtDNA analysis. Our work included dHPLC analysis of both of the HVI and HVII in addition to 5 SNPs (positions 4580, 4793, 5004, 7028, 7202). The approach was to amplify four regions of the mt genome: HVI, HVII, base region 4507-5074(includes SNP positions @ 4580, 4793 and 5004) and base region 6957-7254 (includes SNP positions 7028 and 7202). Minor adjustments in the position of the HVII reverse primer will include a sixth SNP at position 477 in addition to the inclusion of the polymorphic variable $[AC]_5$ repeat that occurs within the base region 515-524. The inclusion of this region along with SNP 477 will further enhance discriminatory power. The increase in length for the respective HVII amplicon will enhance resolution between HVI and HVII when run simultaneously. Although this approach to increasing the discriminatory power of dHPLC analysis of mtDNA has not been completed, we have detected the expected mismatches in all 4 PCR amplified regions. In addition, no false positives/negatives occurred in the small sample set evaluated. The dHPLC approach screened 80 samples for possible matches or mismatches in less than 24 hours. This includes time required for DNA extraction, PCR amplification, heteroduplex formation and dHPLC setup. The work supports the purported utility of dHPLC in the development of better DNA methods.

Initial tests of dHPLC (Varian Helix instrumentation) have resulted in ability to detect mismatches in all four PCR amplified regions. In addition, to date no false positives/negatives have occurred. However, the number of samples tested is small. DNA extraction protocols have been standardized and have provided consistent results. PCR reactions have been standardized as well and both uniplex and multiplex reactions display consistent performance and yield. High throughput dHPLC trials have been

achieved resulting in the ability to screen in excess of 80 samples for possible match or mismatch in less than 24 hours. This includes time required for DN extraction, PCR amplification, heteroduplex formation, and dHPLC setup and run.

Preliminary work on applications of dHPLC to the chromatographic isolation of DNA damaged by covalent linkage with a reactive drug metabolite.

Detection of modified DNA in a criminal exposed to genotoxic chemicals at a crime scene can make a good case for placing the criminal at the scene. Since most chemicals are eliminated from the body within a day after exposure, the time prior to analysis is often critical. Some chemicals persist when bound to DNA or proteins. Examples of chemicals that bind strongly include electrophiles, such as some tear gas components. Electrophiles can also be generated in the body by the action of drug metabolizing enzymes. From another project in our laboratories, we found that a breakdown product formed from smoking methamphetamine is converted by liver enzymes to an electrophilic epoxide that reacts with DNA (26). We found that dHPLC provided an effective method for separating the adduct formed from the parent oligonucleotide.

The mass spectrum of the Dickerson dodecamer oligonucleotide is shown in Figure 4. Analysis of samples from reaction mixtures of *trans*-phenylpropylene oxide and DNA by mass spectrometry showed molecular ions consistent with adducted DNA. Adduction of DNA at three different charge states of −3 (*m/z* 1260), − 4 (*m/z* 945), and − 5 (*m/z* 756) was observed as calculated from the oligo mass calculator program (Figure 5). dHPLC analysis of the reaction mixtures of *trans*-phenylpropylene oxide and DNA showed two prominent adduct peaks (RT 1.6 and 1.8 min, respectively) that were absent in the standards (Figure 6). The oligonucleotide had a retention time of 2.08 min and *trans*-phenylpropylene oxide eluted at 4.57 min.

Discussion

The use of mtDNA for the purpose of genetic identification has become an area of great interest. This is due to copy number, location, and survivability of the mt genome. In cases where genomic DNA has been degraded and subsequent analysis of STR information made difficult or impossible, mtDNA may provide the only means of identification.

Limitations regarding the use of mtDNA for definitive identification do exist. The small size of the mtDNA genome (16,569 bp) does not allow for the power of discrimination obtained through genomic STR analysis. In addition since mtDNA is primarily inherited via maternal linkage, it is not possible to discern between maternal siblings. Although this phenomenon may be advantageous in some scenerios.

Earlier work by LaBerge, *et al.* (23) provided strong evidence of the effectiveness of dHPLC in a forensic setting. However, the published work appears to be limited to the Hypervariable regions I and II which may not provide sufficient discriminatory power.

This is apparent when one considers that 7% of the Caucasians display the same HV haplotype.

Coble, *et al.* (27) identified a panel of forensically relevant SNPs that increase the discriminatory power of mtDNA analysis. Subsequent work by Vallone, *et al.* (26), have incorporated 11 of these SNPs into a multiplex allele-specific primer extension assay. Our current work included dHPLC analysis of both of the HVI and HVII in addition to 5 SNPs identified by Coble et al. (27) (positions 4580, 4793, 5004, 7028, 7202). Currently our approach is to amplify four regions of the mt genome: HVI, HVII, base region 4507-5074(includes SNP positions @ 4580, 4793 and 5004) and base region 6957-7254 (includes SNP positions 7028 and 7202). Minor adjustments in the position of the HVII reverse primer will include a sixth SNP at position 477 in addition to the inclusion of the polymorphic variable $[AC]_5$ repeat that occurs within the base region 515-524. The inclusion of this region along with SNP 477 will further enhance discriminatory power. The increase in length for the respective HVII amplicon will enhance resolution between HVI and HVII when run simultaneously.

The high throughput advantages of dHPLC are evidenced by the fact that we have analyzed 84 samples in less than 24 hr. This includes time used for DNA extraction, PCR amplification, and dHPLC analysis.

Current and future work will tune multiplex PCR/dHPLC conditions in order to achieve conditions that would allow for both maximum throughput and greatest discriminatory power in a single assay to make the method more useful across crime labs. An increase in the number of DNA samples is also needed to maximize validity and significance. This will also allow for an increase the "blinded" studies and attempts to resolve DNA mixtures.

Inclusion of an additional region that includes both SNP 477 and $[AC]_5$ repeat should be considered. This will not only increase discriminatory power, but will also allow greater resolution between the HVI fragment and the larger amplicon incorporating HVII and the above regions (477-525) when attempting to tune dHPLC conditions when running multiplex PCR products.

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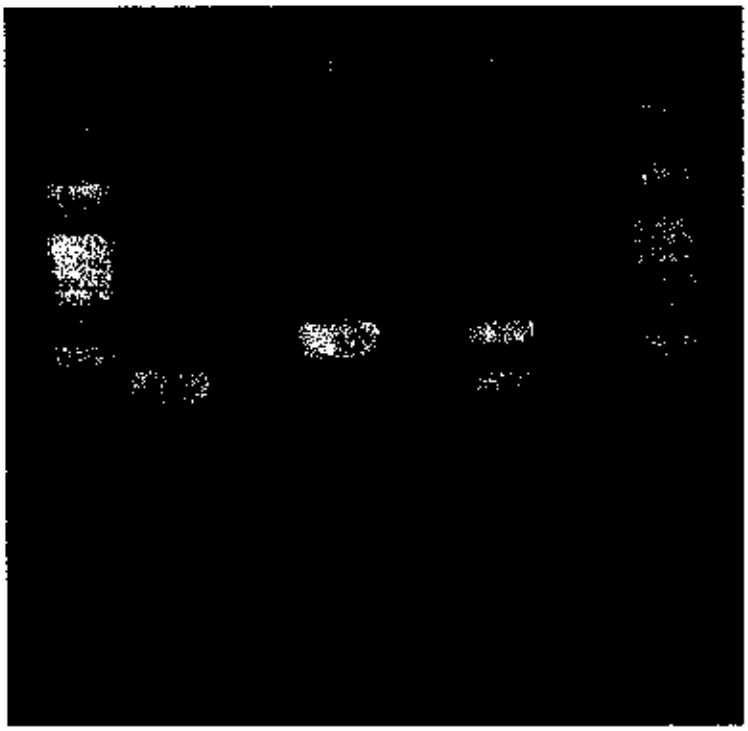

Figure 1: PCR amplification of hypervariable regions from human mtDNA. Primer pairs (Table 1) used alone (uniplex PCR) or in combination (multiplex PCR) to amplify variable segments of human mitochondrial DNA prior to analysis by dHPLC. Hypervariable regions I and II of LaBerge et al. were first chosen. More recently, Vallone et al. identified 11 SNPs in the mitochondrial genome that are highly variable and potentially useful for forensic analysis. Of these, 5 could be included within PCR amplicons suitable for dHPLC analysis using only 2 new and unique primer sets. When amplified, the resulting amplicons were designated as SNP regions I and II, or SRI and SRII, of mtDNA. A gel polymerized from 1% agarose in 1X TBE buffer was used to separate amplified PCR products using standard techniques (Maniatis et al.,). Lanes 1 and 8, 100bp DNA ladder; Lane 2, HVI; Lane 3, HVII; Lane 4, SRI; Lane 5, SRII; Lane 6, multiplex reaction using buccal DNA source; Lane 7, multiplex reaction using hair shaft DNA. Note the poor resolution of the 401 and 433 bp bands (HVI and HVII).

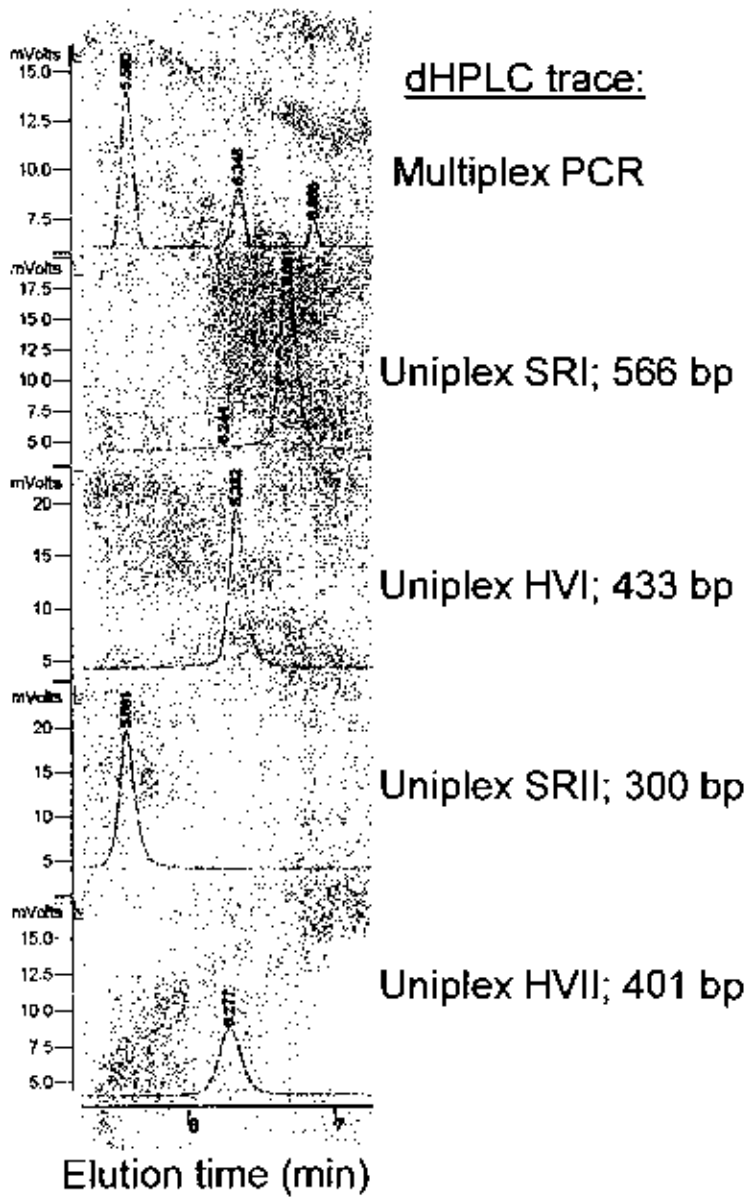

Figure 2: Separation of hypervariable amplicons by dHPLC. The dHPLC traces for each hypervariable amplicon used in the current study are shown individually in the lower four traces. The top trace shows the result of attempts to separate all four amplicons from a single multiplex PCR reaction. Note that, in our hands, the dHPLC was also unable to separate the 401 and 433 bp amplicons.

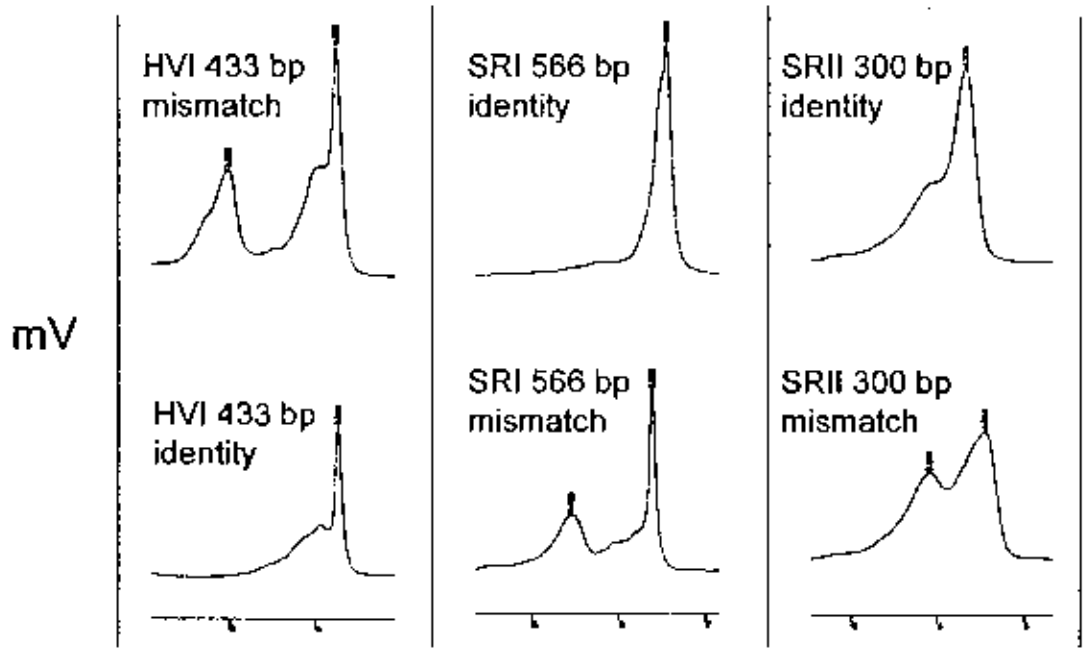

Figure 3: Resolution of identical and non-identical hypervariable amplicons by dHPLC. In order to test the ability of dHPLC to discriminate identical versus non-identical hypervariable amplicons, samples were first randomly assigned a partner. Paired samples were then subjected to re-annealing with self and partner, followed by dHPLC analysis. Representative samples of each resolvable amplicon (minus HVII) are shown with a matching DNA sample as well as a mismatched DNA sample as determined by dHPLC analysis.

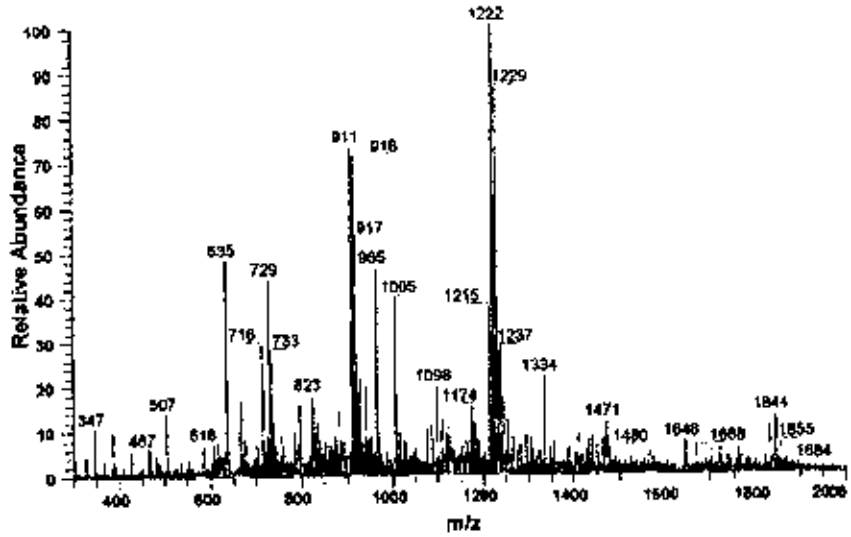

Figure 4: Negative ion electrospray ionization mass spectrum of duplex Dickerson dodecamer oligonucleotide showing -3, -4, and -5 charge states.

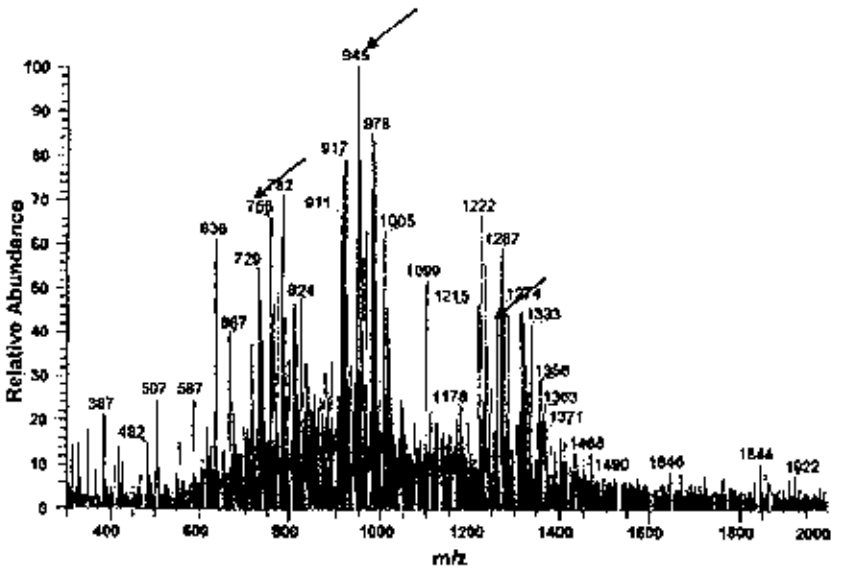

Figure 5: Negative ion electrospray mass spectrum of duplex Dickerson dodecamer oligonucleotide adducted with *trans*-phenylpropylene oxide. Arrows represent the adducts of different charge states.

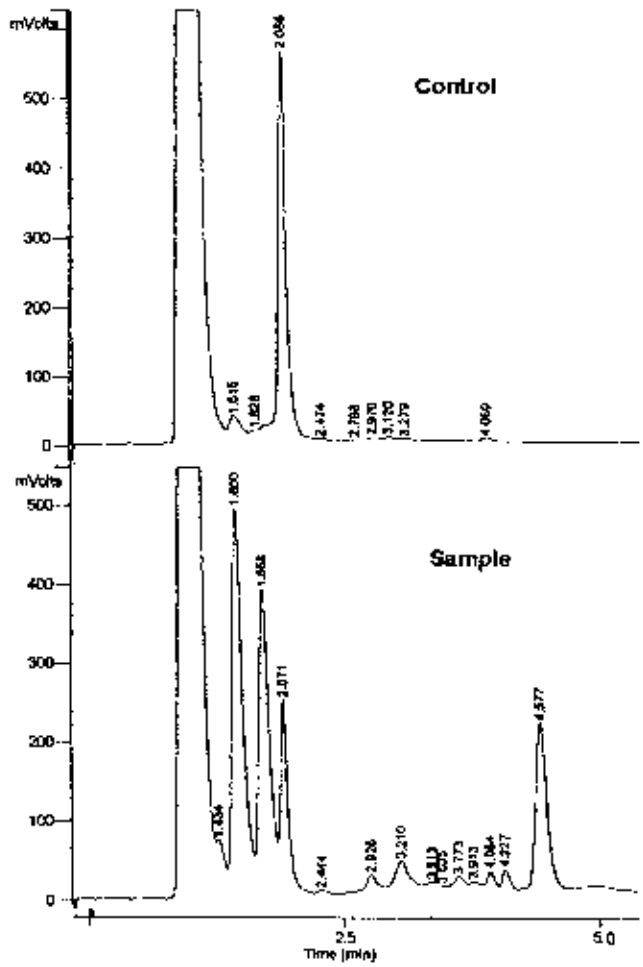

Figure 6: dHPLC chromatograms of Dickerson dodecamer oligonucleotide, R_T=2.086 min (upper chromatogram) and oligonucleotide adducted with *trans*-phenylpropylene oxide (lower chromatogram).